Deletion of ultraconserved elements yields viable mice

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ABSTRACT

Ultraconserved elements have been suggested to retain extended perfect sequence identity between the human, mouse, and rat genomes due to essential functional properties. To investigate the necessities of these elements *in vivo,* we removed four non-coding ultraconserved elements (ranging in length from 222 to 731 base pairs) from the mouse genome. To maximize the likelihood of observing a phenotype, we chose to delete elements that function as enhancers in a mouse transgenic assay and that are near genes that exhibit marked phenotypes both when completely inactivated in the mouse as well as when their expression is altered due to other genomic modifications. Remarkably, all four resulting lines of mice lacking these ultraconserved elements were viable and fertile, and failed to reveal any critical abnormalities when assayed for a variety of phenotypes including growth, longevity, pathology and metabolism. In addition more targeted screens, informed by the abnormalities observed in mice where genes in proximity to the investigated elements had been altered, also failed to reveal notable abnormalities. These results, while not inclusive of all the possible phenotypic impact of the deleted sequences, indicate that extreme sequence constraint does not necessarily reflect crucial functions required for viability.

INTRODUCTION

The use of evolutionary conservation has become a powerful means for identifying functionally important genomic sequences [1,2]. Ultraconserved elements have been defined as a group of extremely conserved sequences that show 100% identity over 200bp or greater between the human, mouse, and rat genomes [3]. This category of extreme evolutionary sequence conservation is represented by 481 sequences in the human genome of which over half show no evidence of transcription. Further analysis of the distribution of these non-coding ultraconserved elements demonstrate that they tend to cluster in regions that are enriched for transcription factors and developmental genes [3], and a limited number of functional studies suggest a role for some of these non-coding elements in gene regulation [4-6].

Several hypotheses have been proposed to explain the extreme sequence constraint of ultraconserved elements including strong negative selective pressure and/or reduced mutation rates [3]. The negative selection hypothesis postulates that crucial functions such as vital gene regulatory information is embedded within these sequences, while the reduced mutation rate hypothesis suggests that these sequences exist in a hyperrepaired or hypomutable state [3]. Recent analysis of human variation in these non-coding ultraconserved elements provides compelling evidence supporting negative selection as contributing to their extreme evolutionary conservation [7]. Furthermore, non-coding ultraconserved elements have also been shown to be significantly depleted in human segmental duplications and copy number variants, suggesting that disruption of their

normal copy number may lead to reduced fitness [8]. In this study, we removed four carefully chosen non-coding ultraconserved elements in the mouse genome to directly explore a functional role for these elements *in vivo*.

RESULTS

Generation and general characterization of ultraconserved knockout mice

To increase the probability of observing an associated phenotype in the ultraconserved null mice, we employed a variety of criteria in selecting the non-coding ultraconserved elements for deletion. We chose elements that showed tissue-specific *in vivo* enhancer activity in a mouse transgenic reporter assay that tended to recapitulate aspects of the expression pattern found in genes that were in their proximity (Figure 1)[6]. Other factors that were taken into account in prioritizing elements for deletion included their proximity to genes whose inactivation and alterations in their expression results in specific phenotypes that we could screen for in the ultraconserved element deletion mice (Table 1). Elements meeting most of these criteria were chosen for removal and included: uc248, uc329, uc467, and uc482 (Figure 1)[3]; representing 222, 307, 731, and 295 base pairs respectively of 100% identity between human, mouse, and rat.

All four non-coding ultraconserved elements were deleted from the mouse genome using standard mouse genetic engineering techniques, and removal was confirmed by PCR and Southern blot hybridization (Supplementary Methods). We first examined each line for the viability of homozygous/hemizygous knockout mice in mixed crosses, and observed that all four lines showed no reduction in the expected number of homozygous/ hemizygous mice that were generated (Table 2). Homozygous matings within the four lines revealed no significant differences in viability and litter size compared to the wild-type littermates (Table 2). We next examined body weight (up to ten weeks of age;

Figure 2), and survival (up to 25 weeks; see Materials and Methods) and found no significant differences compared to the wild-type littermates. Further analysis of a standard panel of 16 different clinical chemistry assays in each of the mouse lines detected but a few small differences compared to the wild-type littermates (Supplementary Figure 1). Expression analysis of genes adjacent to each element by whole-mount in situ hybridization at embryonic day 11.5 (e11.5) revealed no apparent differences between null embryos and their wild-type littermates, except for a moderate reduction in forebrain expression of SRY-box 3 (*Sox3;*Entrez Gene ID 20675) in uc482 null embryos (Supplementary Figure 2). Quantization by real-time PCR suggested a slight reduction in *Sox3* e11.5 head expression that was, however, insignificant (29.63 in wild-types compared to 23.66 in nulls, corresponding to 18S RNA expression; p-value=0.64, unpaired t-test). General pathological analysis of 6-week old mice revealed no distinct differences compared to the wild-type littermates (Supplementary Table 1), with one exception. The exception was one uc329 homozygous male having unilateral renal agenesis. Additional analysis of 102 uc329 homozygous null mice revealed a total of 2 mice (including the initial propositus) with one kidney compared to none within the 30 uc329 wild-type littermates that were screened. Unilateral renal agenesis is estimated to occur in 1 to 1000 live births in humans [9] and is asymptomatic and unassociated with a reduction in survival rate [10]. Possible explanations for unilateral renal agenesis in ~2% of uc329 homozygous null mice in this study include a spontaneous event unassociated with the deleted element or a low penetrance phenotype caused by the absence of this element.

Screens for phenotypes of adjacent genes

In addition to the above general screens, we screened each of these mouse lines for phenotypes specifically associated with the inactivation or dosage abnormality of the genes in proximity to the deleted ultraconserved elements. Ultraconserved element 248 is bracketed by the genes doublesex and mab3-related transcription factor 1 (*DMRT1*; Entrez Gene ID 1761) and doublesex and mab3-related transcription factor 3 (*DMRT3*; Entrez Gene ID 58524) (Figure 1A). In humans, haploinsufficiency due to chromosomal aberrations within this region leads to XY sex reversal [11]. In mice, *Dmrt1* homozygous knockouts exhibit defects in testicular development [12], while *DMRT3* function is unknown. In order to identify the phenotype associated with *Dmrt3* deficiency for these studies we deleted *Dmrt3* from the mouse genome. All *Dmrt3* null homozygous mice died from starvation at two months of age due to dental malocclusions, and in addition some of the males exhibited male sexual development abnormalities (N. Ahituv unpublished results). Based on these results, we extensively phenotyped uc248 homozygous null mice for sexual and dental abnormalities. Pathological analysis of both male and female sexual organs and teeth in 6 week old uc248 null mice revealed no obvious defects (Supplementary Table 1). In addition, heterozygous and homozygous crosses exhibited no reduction in expected homozygous offspring (Table 2).

Ultraconserved element 467, the longest solitary non-coding ultraconserved element in the human genome (731bp), lies inside the last intron of polymerase alpha (*POLA*; Entrez Gene ID 5422) adjacent to the aristaless-related homeobox (*ARX*; Entrez Gene ID

170302) gene (Figure 1C). Mutations in *ARX* in humans leads to a wide range of neurological and sexual development disorders [13,14], while hemizygous *Arx* null male mice die shortly after birth and have small brains and male sexual development abnormalities [15]. In addition, a duplication of this region in mice, caused by insertional mutagenesis, leads to embryonic lethality due to exencephaly accompanied by anophthalmia [16]. Detailed pathological examination of the reproductive organs and neuroanatomical examination of the brains of uc467 null mice revealed no apparent abnormalities (Supplementary Table 1). In addition, the mice showed no obvious differences in the offspring expected from the hemizygous x heterozygous and hemizygous x homozygous crosses (Table 2).

Ultraconserved element 329 lies in the middle of an 80kb intronic region of the hypothetical protein *0610012H03Rik* (GenBank NM_028747) in a region adjacent to the Reticulocalbin 1 (*RCN1*; Entrez Gene ID 5954) gene and two developmental transcription factors, Wilms tumor 1 (*WT1*; Entrez Gene ID 7490*)* and paired box gene 6 (*PAX6*; Entrez Gene ID 5080)(Figure 1B). Mutations in humans in *WT1* and *PAX6* respectively cause Wilms Tumor (OMIM #194070) and type 2 aniridia (OMIM #106210), while chromosomal deletions encompassing all four genes lead to WAGR syndrome (OMIM #194072). Mouse knockouts generated for *Wt1* (OMIM #607102) and *Pax6* (OMIM #607108) have a variety of phenotypes, the most notable being kidney and eye abnormalities respectively. Detailed pathological analysis of the kidneys and eyes of the uc329 null mice revealed no significant differences compared to the wild-type

littermates (Supplementary Table 1), other than the ~2% unilateral renal agenesis discussed above. Clinical chemistry tests revealed slightly higher urea N levels compared to the wild-type littermates (33.16 versus 26.16 mg/dL respectively; p-value 0.032, unpaired t-test; Supplementary Figure 1), while creatinine levels, which are a more specific measure for kidney function, were similar to the wild-type littermates (0.25 versus 0.22 mg/dL respectively; p-value 0.165. unpaired t-test; Supplementary Figure 1).

Ultraconserved element 482 resides in a gene desert between the Atpase class VI type 11C (*ATP11C*; Entrez Gene ID 286410) and SRY-box 3 (*SOX3*; Entrez Gene ID 6658) genes (Figure 1D). Human *SOX3* mutations lead to X-linked mental retardation with isolated growth hormone deficiency [17] and hypopituitarism [18], while *SOX3* dosage defects are suggested to cause hypopituitarism [18] and hypoparathyroidism [19]. In mice, deletion of *Sox3* results in sexual development and pituitary abnormalities [20,21]. Pathological analysis of uc482 null mice reproductive organs revealed no significant abnormalities (Supplementary Table 1), and hemizygous x heterozygous and hemizygous x homozygous crosses exhibited no reduction in expected homozygous/hemizygous offspring (Table 2). Growth hormone abnormalities would be expected to lead to body weight irregularities, none of which were detected (Figure 2). Calcium levels were also normal (Supplementary Figure 1) supporting a lack of marked abnormalities in parathyroid gland function.

DISCUSSION

Based on the compelling evidence that ultraconserved elements are conserved due to functional constraint, it has been proposed that their removal *in vivo* would lead to a significant phenotypic impact [7,8]. Accordingly our results were unexpected. It is likely that our assays were not able to detect dramatic phenotypes that under a different setting, for instance outside the controlled laboratory setting, would become evident. Moreover, possible phenotypes might become evident only on a longer timescale, such as longer generation time. It is also possible that subtler genetic manipulations of the ultraconserved elements might lead to an evident phenotype due to a gain of function type mechanism. All four elements examined in this study demonstrated *in vivo* enhancer activity when tested in a transgenic mouse assay (Figure 1)[6], which would suggest regulatory element redundancy as another possible explanation for the lack of a significant impact following the removal of these specific elements. Just as gene redundancy has been shown to be responsible for the lack of phenotypes associated with many seemingly vital genes knockouts, regulatory sequence redundancy [22] can similarly provide a possible explanation for the lack of a marked phenotype. While our studies have not defined a specific need for the extreme sequence constraints of non-coding ultraconserved elements, they have ruled out the hypothesis that these constraints reflect crucial functions required for viability.

MATERIALS AND METHODS

Generation of ultraconserved element null mice. The basic technology used for gene targeting and screening has been described previously [23]. Briefly, the four selected ultraconserved elements were removed in W4/129S6 mouse embryonic stem cells (Taconic) by standard replacement of a LoxP flanked neomycin cassette. To avoid potential regulatory effects due to the neomycin gene cassette, we subsequently removed it by Cre-mediated recombination of LoxP sites in the ES cells. All positive colonies in each stage were confirmed by PCR and Southern analysis (Supplementary Methods) and then injected into C57BL/6J blastocyst stage embryos. Chimeric mice were subsequently crossed to C57BL/6J mice, generating agouti offspring that were heterozygous/hemizygous for the ultraconserved element deletion and were intercrossed to generate homozygous ultraconserved null mice. Genotyping was carried out using standard PCR techniques (Supplementary Methods).

Survival. 8 males and 8 females from each line and wild-type littermates were analyzed for survival up to 25 weeks. Mice were housed in a temperature-controlled room under a 12-hour light and dark cycle, given free access to water and fed ad libitum on a standard chow. No lethality was observed for any of the strains during the period of study.

Clinical Chemistry. Serum samples from at least 6 males and 6 females at 10-14 weeks of age from each line were analyzed using the automated spectrophotometricchemistry

analyzer Hitachi 917 at Marshfield Laboratories (Wisconsin, USA) following standard protocols.

Whole-mount in situhybridization. Four e11.5 wild-type embryos were analyzed for each gene. Genes that were positive for expression at this time point were further analyzed for expression differences using four homozygous null and four wild-type littermates at e11.5. Briefly, embryos were fixed overnight in 4% paraformaldehyde followed by methanol washes. Whole-mount RNA-in situ-hybridization was carried out using standard protocols [24] with antisensedigoxigenin-labelled riboprobes. The following vectors were used as templates for probes: *Dmrt1* (kind gift from D. Zarkower, University of Minnesota), *Dmrt2* (IMAGE 1248080), *Dmrt3* (IMAGE 6404988), *Pax6* (IMAGE 4504106), *Rcn1* (IMAGE 6414128), *0610012H03Rik* (IMAGE 5042053), *Wt1* (RNA probe 777 from GenePaint.org), *Pola1* (IMAGE 894396, 30063811, 30103897), *Arx* (IMAGE 5707995), *Atp11c* (IMAGE 30843359), *Sox3* (IMAGE 5717161). Stained embryos were analyzed using a LeicaMZ16 microscope and photographed with a LeicaDC480 camera.

Real-time quantitative PCR. Total RNA was extracted using TRIzol reagent (Invitrogen) from the heads of four uc482 homozygous/hemizygous null and four wild-type littermates at e11.5, and pooled separately. Following reverse transcription with SuperScript™ First-Strand Synthesis System (Invitrogen), real-time PCR was performed using *Sox3* gene specific primers (Fwd:agcgcctggacacgtacac, Rev: atgtcgtagcggtgcatct), QuantumRNA Universal 18S (Ambion), and the SYBR Green PCR Master Mix (Applied

Biosystems) on a 7500 Fast Real-Time PCR System (Applied Biosystems). All procedures and calculations were carried out according to manufacturer's recommendations.

Necropsy and pathology examination. 2 male and 2 female 6 week old mice from each knockout line and wild-type littermates were submitted to the Comparative Pathology Laboratory at UC Davis. Tissues were fixed in 10% phosphate buffered formalin for at least 24 hours and processed using routine methods to Hematoxylinand Eosin stained slides and subsequently analyzed for any abnormalities.

REFERENCES

1. Boffelli D, Nobrega MA, Rubin EM (2004) Comparative genomics at the vertebrate extremes. Nat Rev Genet 5: 456-465.
2. Dermitzakis ET, Reymond A, Antonarakis SE (2005) Conserved non-genic sequences - an unexpected feature of mammalian genomes. Nat Rev Genet 6: 151-157.
3. Bejerano G, Pheasant M, Makunin I, Stephen S, Kent WJ, et al. (2004) Ultraconserved Elements in the Human Genome. Science 6: 6.
4. Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, et al. (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125: 315-326.
5. Lee TI, Jenner RG, Boyer LA, Guenther MG, Levine SS, et al. (2006) Control of developmental regulators by Polycomb in human embryonic stem cells. Cell 125: 301-313.
6. Pennacchio LA, Ahituv N, Moses AM, Prabhakar S, Nobrega MA, et al. (2006) In vivo enhancer analysis of human conserved non-coding sequences. Nature 444: 499-502.
7. Drake JA, Bird C, Nemesh J, Thomas DJ, Newton-Cheh C, et al. (2006) Conserved noncoding sequences are selectively constrained and not mutation cold spots. Nat Genet 38: 223-227.
8. Derti A, Roth FP, Church GM, Wu CT (2006) Mammalian ultraconserved elements are strongly depleted among segmental duplications and copy number variants. Nat Genet 38: 1216-1220.

9. Cascio S, Paran S, Puri P (1999) Associated urological anomalies in children with unilateral renal agenesis. J Urol 162: 1081-1083.

10. Argueso LR, Ritchey ML, Boyle ET, Jr., Milliner DS, Bergstralh EJ, et al. (1992) Prognosis of patients with unilateral renal agenesis. Pediatr Nephrol 6: 412-416.

11. Muroya K, Okuyama T, Goishi K, Ogiso Y, Fukuda S, et al. (2000) Sex-determining gene(s) on distal 9p: clinical and molecular studies in six cases. J Clin Endocrinol Metab 85: 3094-3100.

12. Raymond CS, Murphy MW, O'Sullivan MG, Bardwell VJ, Zarkower D (2000) Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. Genes Dev 14: 2587-2595.

13. Sherr EH (2003) The ARX story (epilepsy, mental retardation, autism, and cerebral malformations): one gene leads to many phenotypes. Curr Opin Pediatr 15: 567-571.

14. Kato M, Das S, Petras K, Kitamura K, Morohashi K, et al. (2004) Mutations of ARX are associated with striking pleiotropy and consistent genotype-phenotype correlation. Hum Mutat 23: 147-159.

15. Kitamura K, Yanazawa M, Sugiyama N, Miura H, Iizuka-Kogo A, et al. (2002) Mutation of ARX causes abnormal development of forebrain and testes in mice and X-linked lissencephaly with abnormal genitalia in humans. Nat Genet 32: 359-369.

16. Cunningham D, Xiao Q, Chatterjee A, Sulik K, Juriloff D, et al. (2002) exma: an X-linked insertional mutation that disrupts forebrain and eye development. Mamm Genome 13: 179-185.

17. Laumonnier F, Ronce N, Hamel BC, Thomas P, Lespinasse J, et al. (2002) Transcription factor SOX3 is involved in X-linked mental retardation with growth hormone deficiency. Am J Hum Genet 71: 1450-1455.

18. Woods KS, Cundall M, Turton J, Rizotti K, Mehta A, et al. (2005) Over- and underdosage of SOX3 is associated with infundibular hypoplasia and hypopituitarism. Am J Hum Genet 76: 833-849.

19. Bowl MR, Nesbit MA, Harding B, Levy E, Jefferson A, et al. (2005) An interstitial deletion-insertion involving chromosomes 2p25.3 and Xq27.1, near SOX3, causes X-linked recessive hypoparathyroidism. J Clin Invest 115: 2822-2831.

20. Weiss J, Meeks JJ, Hurley L, Raverot G, Frassetto A, et al. (2003) Sox3 is required for gonadal function, but not sex determination, in males and females. Mol Cell Biol 23: 8084-8091.

21. Rizzoti K, Brunelli S, Carmignac D, Thomas PQ, Robinson IC, et al. (2004) SOX3 is required during the formation of the hypothalamo-pituitary axis. Nat Genet 36: 247-255.

22. Frazer KA, Tao H, Osoegawa K, de Jong PJ, Chen X, et al. (2004) Noncoding sequences conserved in a limited number of mammals in the SIM2 interval are frequently functional. Genome Res 14: 367-372.

23. Nagy A, Gertsenstein M, Vintersten K, Behringer R (2002) Manipulating the Mouse Embryo: A Laboratory Manual: Cold Spring Harbor Laboratory Press.

24. Hargrave M, Bowles J, Koopman P (2006) In situ hybridization of whole-mount embryos. Methods Mol Biol 326: 103-113.

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FIGURE LEGENDS

Figure 1. Schematic of the human genomic location of the four ultraconserved elements that were deleted. **a**, uc248 region. **b**, uc329 region. **c**, uc467 region. **d**, uc482 region. A black oval represents each ultraconserved element while the above embryos represent observed positive enhancer activities captured through transgenic mouse testing at e11.5 for that element[6]. Stained embryos in boxes represent whole-mount *in situ* hybridizations of the specific gene at e11.5 (genes without stained embryos were negative for this assay at this time point).

Figure 2. Growth curves of each ultraconserved element knockout mouse strain. Error bars indicate standard deviation.

Supplementary Figure 1. Clinical chemistry analysis in ultraconserved knockout mice. Bar charts representing the average parameters for each line, error bars indicate standard deviation. An asterisk indicates a p-value <0.05 in an unpaired t-test compared to the wild-type littermates.

Supplementary Figure 2. Whole-mount *in situ* hybridization analysis of homozygous/hemizygous knockouts and wild-type littermates. Representative embryos for each gene that is positive for this assay at e11.5 are shown. The red arrows indicate the *Sox3* forebrain expression in uc482 embryos.

Supplementary Table 1. Summary of pathological analysis in ultraconserved knockout mice.

Supplementary Methods

Generation of ultraconserved element null mice

Homologous arms were amplified by PCR (see primer tables below for each element) from DNA extracted from W4/129S6 mouse embryonic stem cells (Taconic) and cloned into the ploxPN2T vector. ploxPN2T was modified from pPN2T [1] by replacing the existing neomycin selection cassette with a neomycin selection cassette flanked by loxP sites for positive selection and two hsv-tk cassettes for negative selection. Constructs were linearized and 20 μg were electroporated into W4/129S6 mouse embryonic stem cells. The electroporated cells were selected under G418 (160 μg/ml) and 0.2 μM FIAU for a week. Colonies that survived were picked and expanded on 96-well plate and screened both by PCR with primers outside but flanking the homologous arm, and by Southern blot hybridization with probes targeting the vicinity of the homologous arms. To avoid potential regulatory effects due to the neomycin gene cassette, we next removed it by Cre-mediated recombination of LoxP sites in the ES cells. Clones that were correctly targeted were electroporated with 20 μg of the Cre recombinase-expressing plasmid TURBO-Cre (Obtained from Dr. Timothy J. Ley of the Embryonic Stem Cell Core at the Siteman Cancer Center, Washington University Medical School). Clones positive for Neo removal were screened both by PCR (primers and gels for each element below) and Southern blot hybridization (gel results below for each element), and checked for G418 sensitivity. Positive colonies were injected into C57BL/6J blastocyst stage embryos. Chimeric mice were subsequently crossed to C57BL/6J mice, generating

agouti offspring that were heterozygous/hemizygous for the ultraconserved element deletion and were intercrossed to generate homozygous ultraconserved null mice.

Genotyping

Genomic DNA was extracted from a 1-cm section of tail that was incubated overnight in lysis buffer (containing 50mM Tris-HCl pH 8.0, 1mM EDTA, 1% SDS, 20mM NaCl and 1mg/ml Proteinase K) at 55 degrees Celsius. Genotyping was performed by PCR, with primers outside the deleted segments and within the targeting vector (see primer tables below for each element).

Figure legend for genomic targeting and screening below.

Design of ultraconserved deletions displaying the wild-type, the Neo insertion (+Neo) and the Neo deletion (DEL) allele for each element (figures not drawn to scale). The points of integration of target vector homology arms are shown, primers (P2/P3.fwd and P2/P3.rev) used to screen embryonic stem (ES) cells by PCR for the presence of the Neo cassette and the Neo cassette deleted cells are indicated by arrows flanking the deleted segment. The probe used in the Southern hybridization for identification of embryonic stem cells carrying the deletion is shown as a striped box, together with the restriction sites. The sequence that was deleted, the expected DNA sizes for the Southern hybridization and PCR, the Southern hybridization and PCR gel results of each allele, and the primers that were used are shown following the figure.

Table 1. Mouse knockout and dosage sensitive phenotypes for genes adjacent to the deleted ultraconserved elements.

ultra					
uc248	Gene	***Dmrt1***	***Dmrt3***	***Dmrt2***	
	Type	transcription factor	transcription factor	transcription factor	
	KO[a]	Male sexual development [12]	Male sexual development, dental malocclusions	Not reported	
	Dosage[b]	Deletion of region in humans results in defective sexual determination [11]			
uc329	Gene	***Pax6***	***Rcn1***	***0610012H03Rik***	***Wt1***
	Type	transcription factor	calcium-binding	unknown	transcription factor
	KO	Eye, lethality, CNS[c], craniofacial, pituitary, pancreas (OMIM #607108)	Not reported	Not reported	Kidney, lethality, sexual development, mesothelium¸heart, lungs. (OMIM #607102)
	Dosage	Deletion of region in humans results in WAGR syndrome (OMIM #194072)			
uc467	Gene	***Pola1***	***Arx***		
	Type	DNA polymerase	transcription factor		
	KO	Not reported	Lethality, smaller brains, male sexual development[15]		
	Dosage	Duplication of this region in mice leads to lethality due to due to exencephaly and anophthalmia [16]			
uc482	Gene	***Atp11c***	***Sox3***		
	Type	ATPase	transcription factor		
	KO	Not reported	Sexual development, pituitary function [20,21]		
	Dosage		Hypopituitarism [18], hypoparathyroidism[19]		

[a]Knockout mouse phenotype.
[b]Dosage or expression sensitive abnormalities.
[c]Central nervous system.

Table 2. Summary of observed offspring from various ultraconserved targeted mouse crosses.

Het(+/-) x Het(+/-)	**Wt(+/+)**	**Het(+/-)**	**Hom(-/-)**		**p-value[a]**
uc248	77	192	99		0.0004
uc329	128	224	114		0.0629
Hem(X^-/Y) x Het(X^+/X^-)	**Wt(X^+/Y)**	**Hem(X^-/Y)**	**Het(X^+/X^-)**	**Hom(X^-/X^-)**	**p-value[b]**
uc467	80	80	82	71	0.7861
uc482	91	121	93	111	0.0024
Hom(-/-)/Hem(X^-/Y) x Hom (-/-)/(X^-/X^-)	**Birth/Weaning**	**Males**	**Females**	**Litter size[c]**	**p-value[d] (litter)**
uc248	176/173	93	80	7.33	0.64
uc329	225/220	123	97	7.25	0.43
uc467	201/195	103	92	8.04	0.65
uc482	269/254	126	128	8.67	0.16
wild-type	239/235	106	129	7.70	

[a] Chi-Square test for expected 1:2:1 ratio.
[b] Chi-Square test for expected 1:1:1:1 ratio.
[c] Average litter size at birth.
[d] Unpaired t-test of litter size compared to wild-type.

ABBREVIATIONS

aristaless-related homeobox (*ARX*)

Atpase class VI type 11C (*ATP11C*)

Central nervous system (CNS)

Doublesex and mab3-related transcription factor 1 (*DMRT1*)

Doublesex and mab3-related transcription factor 3 (*DMRT3*)

Embryonic day 11.5 (e11.5)

Milligram per deciliter (mg/dL)

Online mendelian inheritance in man (OMIM)

Paired box gene 6 (*PAX6*)

Polymerase alpha (*POLA*)

Polymerase chain reaction (PCR)

Reticulocalbin 1 (*RCN1*)

Ribonucleic acid (RNA)

SRY-box 3 (*SOX3*)

Wilms tumor 1 (*WT1)*